Liposomal antioxidants provide prolonged protection against acute respiratory distress syndrome

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Background. *We have previously shown that N-acetylcysteine (NAC), an antioxidant, in the resuscitation fluid after shock prevents lung injury in response to lipopolysaccharide (LPS) by inhibiting chemokine generation by alveolar macrophages in the lung. However, the protection was short-lived. We hypothesized that liposomal (Lip) NAC delivered intratracheally might be delivered directly to the target cells and exert prolonged effect.*

Methods. *Sprague-Dawley rats were bled to a blood pressure of 40 mm Hg for 1 hour and resuscitated with shed blood and equal volume of Ringer's lactate. In some studies 500 mg/kg NAC was included in the resuscitation fluid. Thirty minutes later, 150 μl LipNAC (9.4 mg/kg NAC) was given intratracheally. One hour and 18 hours after resuscitation, LPS (30 μg/kg) or saline was given intratracheally. Lung injury was assessed by permeability to ^{125}I-albumin, bronchoalveolar lavage neutrophils and lung myeloperoxidase. The cytokine-induced neutrophil chemoattractant (CINC) expression in the lung was assessed by Northern blot.*

Results. *At the early time point, both NAC and LipNAC protected the lung with the effects in significantly reducing the increases in transpulmonary albumin flux, neutrophil influx and myeloperoxidase in the lungs of shock/LPS rats. However, by the late time point, only LipNAC retained its salutary effect. This correlated well with persistent ability to prevent CINC increase. In addition, Lipα-tocopherol (α-T) and LipNAC/α-T were tested and determined to be effective to protect the lung.*

Conclusions. *Liposomal encapsulation of antioxidants at low dose provides long lasting protection against acute respiratory distress syndrome after shock. This may represent a novel treatment approach. (Surgery 2000;128:332-8.)*

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ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) occurs in up to 50% of patients after major trauma and is a significant contributor to the morbidity and mortality in this patient population.[1] The development of this clinical syndrome, characterized by hypoxemia, reduced lung compliance and the presence of diffuse lung infiltrates on chest x-ray, is related primarily to the sequestration of neutrophils (PMNs) in the lung followed by their release of various tissue toxic molecules. Among these, reactive oxygen species elaborated, in part, by PMNs in the inflamed lung are known to be generated during ARDS.[2] Their contribution to tissue injury is best demonstrated by the ability of antioxidants to prevent injury in various experimental models.[3] Recent studies have also provided support for the concept that these molecules may play a physiologic role in the regulation of cell function. Oxidants, such as hydrogen peroxide, have been shown to induce the expression of several of the early response genes and to activate the transcription factors AP-1 and NF-κB.[4,5] Antioxidants prevent activation of several of the inflammatory genes by preventing NF-κB–dependent activation of transcription. Antioxidant therapy therefore might act by 2 separate mechanisms, by scavenging toxic reactive oxygen intermediates and by impairing cell signaling processes in cells

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with lessening of the local response to an inflammatory stimulus.

Hemorrhagic shock after trauma is thought to contribute to the pathogenesis of ARDS by priming the immune system and thus enhanced organ injury in response to a second, often trivial inflammatory stimulus, the so-called two-hit model.[6] For example, ischemia-reperfusion has been shown to prime circulating PMNs for increased superoxide production with the potential result that PMN-mediated cytotoxicity might be enhanced once cells are sequestered in the lung.[7] Furthermore, shock may render the lung more susceptible to inflammatory stimuli. Previous work from our group has shown that rodents resuscitated from hemorrhagic shock exhibited enhanced lung PMN sequestration and lung injury in response to a subsequent challenge with intratracheal lipopolysaccharide (LPS).[8] With this model, we demonstrated that increased expression of the lung chemokine, cytokine-induced neutrophil chemoattractant (CINC), was primarily responsible for increased leukosequestration and lung injury. Consistent with a central role for oxidant-mediated priming in this effect, the inclusion of the antioxidant *N*-acetylcysteine (NAC) in the resuscitation fluid prevented the rise in CINC and the consequent PMN sequestration and thus precluded lung injury. The duration of the protective effect of the single parenteral dose of soluble NAC in the resuscitation fluid, however, was not evaluated.

Liposomes are artificially prepared phospholipid vesicles with amphipathic features. Both hydrophilic and lipophilic molecules can be encapsulated in liposomes through incorporation of each into the aqueous space or the lipid bilayers, respectively. Liposome technology is suggested to be a desirable drug delivery system because these systems are biocompatible, biodegradable, and relatively nontoxic. In the present study, we hypothesized that encapsulation of antioxidants in liposomes might promote their therapeutic potential against oxidant-induced lung injury, presumably by facilitating the intracellular uptake of antioxidant molecules and extending the half-lives of the encapsulated antioxidants. To test this possibility, we developed liposome encapsulated NAC (LipNAC) and, in addition, liposome encapsulated α-tocopherol (Lipα-T) and liposome encapsulated NAC/α-tocopherol (LipNAC/α-T). In the present studies we showed that, whereas parenteral soluble NAC provided transient protection against LPS-induced lung injury, the instillation of low dose intratracheal liposome-associated antioxidants provided prolonged protection against ARDS after shock. This observation may suggest an effective adjunctive treatment strategy for oxidant-induced lung injury.

MATERIAL AND METHODS

Animal model of hemorrhagic shock and lung injury. The animal model was performed as described previously.[8] Briefly, Male Sprague-Dawley rats (300 to 350 g; Charles River, St Constant, Quebec, Canada) were anesthetized with ketamine and xylazine administered intraperitoneally. The right carotid artery was cannulated for monitoring of mean arterial pressure, blood sampling and resuscitation. Hemorrhagic shock was initiated by blood withdrawal and reduction of the mean arterial pressure to 40 mm Hg. The mean arterial pressure was then maintained at 40 mm Hg for 60 minutes. Animals were resuscitated by transfusion of the shed blood and Ringer's lactate in a volume equal to that of shed blood. In some studies, animals received NAC (0.5 g/kg) via the artery before the infusion of Ringer's lactate. The catheter was then removed, the carotid artery ligated, and the cervical incision closed. Sham animals underwent the same surgical procedures, but hemorrhage was not induced.

Thirty minutes after resuscitation, the endotracheal instillation of liposome-associated NAC and/or α-T was performed. The liposomal suspension was delivered to the lung via the trachea by a PE-50 polyethylene tubing connected to a 25-gauge epidural catheter. All animals received 150 μl of liposomal preparation that contained 9.4 mg/kg body weight of NAC or α-T and then underwent 20 mechanically ventilated breaths with a rodent ventilator. Control groups in some studies included intratracheal administration of liposomes without incorporated antioxidants or low dose antioxidant that was not incorporated into liposomes. One hour or 18 hours after hemorrhage-resuscitation, either LPS (*Escherichia coli* O11B4, 30 μg/kg in 200 μL saline) or saline (SAL) alone was administered intratracheally. Animals were killed in 4 hours by pentobarbital overdose.

Preparation of liposome-associated antioxidants. Liposome-associated antioxidants were prepared as described previously.[9] Liposomes containing NAC were prepared from a mixture of dipalmitoylphosphatidylcholine (DPPC) and cholesterol (both from Avanti Polar Lipids, Alabasier, Ala) in a 7:3 molar ratio with NAC entrapped, and liposomes containing α-T were prepared from a mixture of DPPC:α-T:cholesterol in a 7:2:1 with or without NAC entrapped. Liposomal vesicle size was determined with the use of a Coulter N4SD parti-

cle-size analyzer. The vesicle size of LipNAC and LipNAC-α-T was 337.3 ± 15.0 nm and 477.0 ± 7.00 nm, respectively.

Bronchoalveolar lavage. For bronchoalveolar lavage (BAL), the lungs were lavaged via an intratracheal angiocatheter with cold phosphate-buffered saline (PBS; 8 mmol/L sodium phosphate, 2 mmol/L potassium phosphate, 0.14 mol/L sodium chloride, 0.01 mol/L potassium chloride, pH 7.4 with 0.1 mmol/L ethylenediaminetetraacetic acid). PBS was instilled in 10-mL aliquots and gently withdrawn with a 10-mL syringe to a total volume of 40 mL.[10]

BAL fluid was centrifuged at 300*g* for 10 minutes to pellet cells. Supernatant was discarded, and the pelleted cells were resuspended in a small volume of serum-free Dulbecco's modified Eagle's medium (Gibco, Burlington, Ontario, Canada). Total cell counts were determined on a grid hemocytometer. Differential cell counts were enumerated on cytospin-prepared slides that were stained with Wright-Giemsa stain. A total of 500 cells were counted in cross-section per sample, and the number of PMNs was calculated as the total cell count times the percentage of the respective cell type in the BAL fluid (BALF) sample.

Assessment of transpulmonary albumin flux. Transpulmonary albumin flux was assessed by injecting 10 μCi ^{125}I-albumin in a total volume of 0.2 mL SAL into the tail vein immediately after intratracheal LPS or SAL.[10] Four hours after LPS, 1 mL blood was withdrawn by cardiac puncture for counting cpm. After exsanguination, lungs were perfused via a cannula in situ with 10 mL PBS. The perfused PBS was withdrawn gently and aliquoted into 1 mL/tube for counting cpm. The transpulmonary albumin flux was normalized to blood cpm as follows:

$$\text{Transpulmonary albumin flux} = \frac{\text{BALF cpm/mL}}{\text{Blood cpm/mL}}$$

Northern blot analysis. Total RNA from lungs was obtained by using the guanidium-isothiocyanate method.[11] Briefly, lungs were harvested from animals and immediately frozen in liquid nitrogen. Lungs were then thawed and homogenized in 10 mL 4M guanidine-isothiocyanate containing 25 mmol/L sodium citrate, 0.5% sarcosyl, and 100 mmol/L β-mercaptoethanol. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was carried out by using a [^{32}P]adenosine triphosphate–end-labeled 30-base oligonucleotide probe for CINC with the sequence 5′-GCGGCATCACCTTCAAACTCTG-GATGTTCT-3′, which is complementary to nucleotides 134 to 164 of CINC cDNA.[12] Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. Blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which is a ubiquitously expressed housekeeping gene, to control for loading.[13] Expression of mRNA was quantitated by using a phosphoimager and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, Calif) and was normalized to the G3PDH signal.

Measurement of myeloperoxidase in the lung. The activity of myeloperoxidase in sonicated whole lung homogenates was carried out by using a kit (R&D Systems, Minneapolis, Minn) according to the manufacturer's directions.

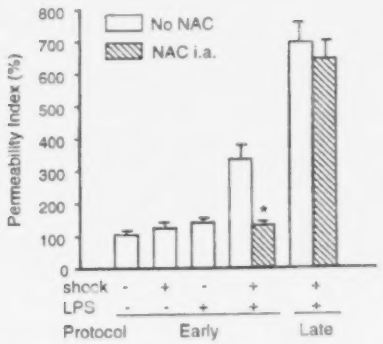
Statistics. The data are presented as mean ± standard error of n determinations as indicated in the figure legends. Data were analyzed by one-way analysis of variance; post hoc testing was performed by using the Bonferroni modification of the *t* test. When individual studies are demonstrated, these are representative of at least 3 independent studies.

RESULTS

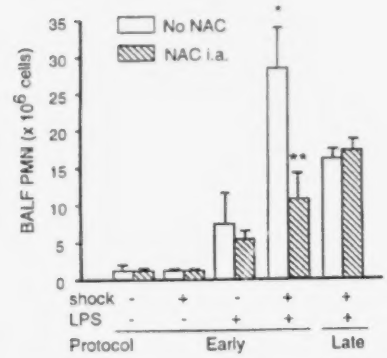
Effect of parenteral NAC on LPS-induced lung injury after shock.

Two protocols for LPS administration were used in these studies. In the "early" protocol, LPS was given 1 hour after the end of shock/resuscitation and lung parameters were measured 4 hours later. In the "late" protocol, LPS was administered 18 hours after the end of resuscitation, with measurements of lung injury being performed 4 hours later. As previously reported by our group, LPS administration 1 hour after resuscitation significantly enhanced both transpulmonary albumin flux (Fig 1, *A*) and BALF PMN count (Fig 1, *B*), consistent with the priming effect of shock. When NAC (0.5 mg/kg) was included in the resuscitation fluid and LPS was given 1 hour after end resuscitation, both lung permeability and PMN counts were significantly reduced in the shock/LPS group when assessed 4 hours later (Fig 1, *A* and *B*, respectively, early protocol). When LPS was administered according to the late protocol, the priming for both increased lung permeability and PMN numbers persisted (Fig 1, *A* and *B*, respectively). However, at this delayed time point, NAC included in the resuscitation fluid was no longer protective. Thus, the protective effect of NAC appeared to be transient in this model.

We have previously shown that enhanced elaboration of CINC by alveolar macrophages in the



A



B

Fig 1. A, Effect of soluble NAC on transpulmonary albumin flux 4 h after treatment with LPS or SAL. NAC (0.5g/kg, intra-arterial) was given during resuscitation and LPS (30 μg/kg) was administered intratracheally 1 (Early) and 18 h (Late) after resuscitation. Data represent the mean ± SEM of studies performed in 5 rats in each group. *Asterisk*, $P < .01$ vs sham/SAL, shock/SAL and sham/LPS groups. *Two asterisks*, $P < .01$ vs shock/LPS group. B, Effect of NAC on BAL PMN counts in sham or shock rats 4 h after LPS or SAL. Data represent the mean ± SEM of studies performed in 4 rats in each group.

shock/LPS animals contributed significantly to the augmented lung neutrophilia and injury in this group.[8] As shown in Figure 2, NAC markedly abrogated the shock-induced priming for increased CINC mRNA expression in response to LPS, when studied early after shock/resuscitation (early group). By contrast, CINC mRNA levels were unaffected by NAC in the resuscitation fluid when studied after delayed administration of LPS (late group). These findings are consistent with the lack of protection exhibited by NAC at this delayed time point.

Effect of liposomal formulation of antioxidants on lung injury. Figure 3 shows the effect of various

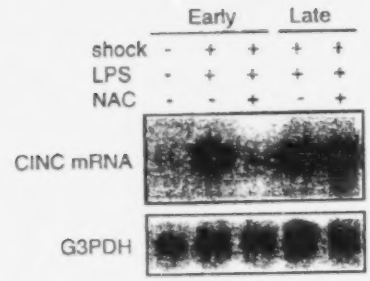


Fig 2. Representative Northern blot from 4 independent studies showing effect of NAC (0.5 g/kg, i.a.) on expression of CINC mRNA extracted from lung tissue of shock and sham animals 4 h after intratracheal LPS. NAC significantly prevented the increase in shock-primed CINC mRNA expression in response to LPS 5 h after NAC (early group). By contrast, the rise of CINC expression was not attenuated by parenteral soluble NAC 22 h later (Late). G3PDH mRNA bands are shown as evidence of comparable loading.

liposome-associated preparations of antioxidants on lung permeability index with the late protocol, ie, when LPS was given at t = 18 hours after resuscitation. In these studies the intratracheal liposomal antioxidants were administered at the end of resuscitation. Compared with no treatment, intratracheal LipNAC significantly reduced transpulmonary albumin flux. Empty liposomes alone did not alter the lung injury induced by shock/LPS. In addition, free NAC given intratracheally at this time similarly had no protective effect (data not shown). α-Tocopherol is a potent hydrophobic antioxidant. When incorporated into a liposomal formulation, it may be delivered directly to the lung.[14] Lip α-T also lessened lung permeability, whereas the combination of antioxidants was no better than LipNAC alone (Fig 3). These data suggest that liposomal encapsulation might prolong the effect of NAC.

Liposomal formulations of antioxidants also reduced PMN accumulation in the lung after shock/LPS. As shown in Figure 4, LPS after antecedent resuscitated shock caused a 12.4-fold increase in PMNs in BALF and a 14.4-fold increase in myeloperoxidase activity (Fig 4, *A* and *B*, respectively) compared with sham/SAL treatment, measured at 22 hours after end resuscitation. Lung neutrophilia was reduced in all 3 liposomal antioxidants treatment groups, although not to the extent seen when NAC was administered by the parenteral route (compare magnitude of reduction in Fig 1 to Fig 4).

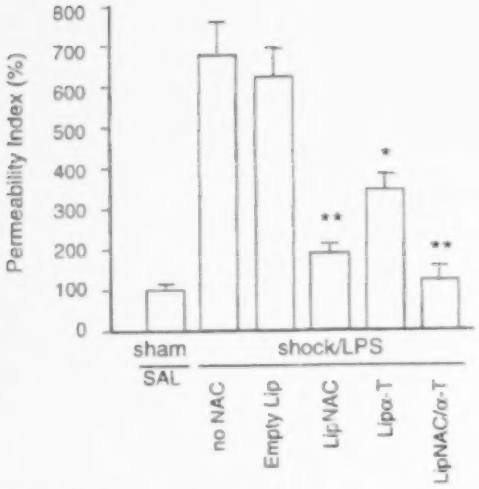


Fig 3. Prolonged effect of liposomal antioxidants on transpulmonary albumin flux in shock/LPS animals. Intratracheal liposomal antioxidants, as labeled, and empty liposome were given at the end of shock/resuscitation, followed by intratracheal LPS 18 h later. The lung permeability index was assessed 4 h after LPS. Data represent the mean ± SEM, n = 7 animals per group. $^{*}P < .05$ and $^{**}P < .01$ vs shock/LPS no NAC group.

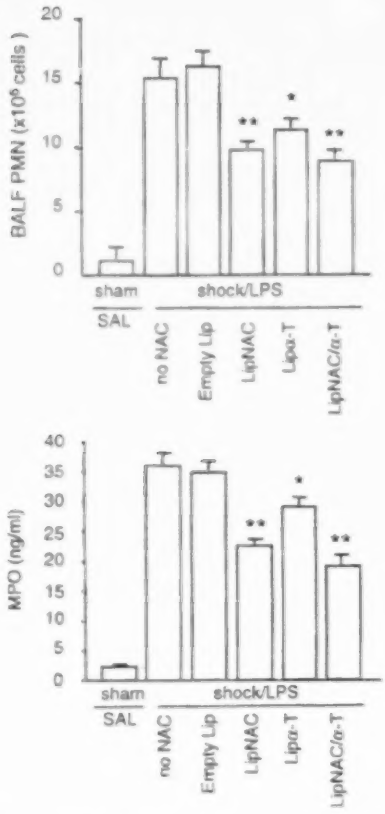


Fig 4. A. Effect of various liposome-associated preparations of antioxidants on PMN counts in BALF with the Late protocol. BAL fluid was collected from the rats 22 h after administration of liposomal antioxidants and 4 h after treatment of LPS. Data represent the mean ± SEM, n = 7 rats per group. **B.** Effect of liposomal antioxidants on lung myeloperoxidase *(MPO)*. Timeline for administration of liposomal antioxidants, LPS and sampling is same as described in **A**. Data represent the mean ± SEM, n = 7 animals per group. $^{*}P < .05$ and $^{**}P < .01$ vs shock/LPS no NAC group.

Figure 5, *A* is a representative Northern blot of CINC mRNA levels in whole lung tissue measured according to the late protocol. Lung CINC mRNA levels in the shock/LPS were elevated compared with sham/LPS when LPS was given 18 hours after end resuscitation and tissues recovered 4 hours later. All 3 liposomal antioxidant formulations decreased CINC mRNA in shock/LPS animals, while having little effect in LPS-treated animals. Figure 5, *B* shows densitometric analysis of CINC mRNA normalized for G3PDH. CINC mRNA levels were increased ~32-fold after shock/LPS treatment compared with Sham/SAL, whereas liposomal NAC reduced shock/LPS-induced CINC mRNA levels by ~70%.

DISCUSSION

Neutrophils sequestered in the lung play a central role in the pathogenesis of ARDS. Lung PMN accumulation seen in ARDS is thought to occur as a result of a cascade of cellular events initiated by either infectious or noninfectious inflammatory stimuli. Local activation of resident cells in the lung interstitium and alveolus, primarily macrophages, leads to elaboration of several proinflammatory cytokines such as tumor necrosis factor and interleukin-1, as well as chemokines including interleukin-8 and macrophage inflammatory protein-2. These mediator molecules act in concert to promote PMN sequestration by activating endothelial cell adhesion molecule expression and to induce migration of PMNs into the interstitium and alveolar space, where they propagate inflammation and injury through the release of reactive oxygen species and proteolytic enzymes.[15] Our previous studies demonstrated that resuscitated hemorrhagic shock rendered rodents more susceptible

to lung injury in response to a second inflammatory stimulus.[8] The finding that augmented CINC elaboration by alveolar macrophages derived from shocked animals was central to the enhanced lung injury is consistent with the hypothesis that shock/resuscitation is able to prime host cells. Furthermore, because NAC inclusion in the resuscitation fluid prevented CINC expression and lung PMN accumulation, it appeared that priming was related to oxidant-mediated effects, possibly via the xanthine-xanthine oxidase system, generated by global ischemia-reperfusion. In the present studies, we demonstrated that addition of NAC to the resuscitation fluid provided protection for lung injury that is limited in duration. By contrast, intratracheal liposomal antioxidants exerted prolonged protection for up to 22 hours after the end of resuscitation. This effect was unlikely due to an LPS-chelating effect of lipid formulations, because empty liposomes are not protective. Similarly, intratracheal administration of unencapsulated NAC did not exert protection, suggesting the route per se was not responsible for the salutary effect. Finally, the liposomal formulations exerted relatively high therapeutic efficacy with low dose of antioxidants required compared with that shown effective by parenteral administration. Considered together with the findings that NAC levels in the lung after their liposomal administration are high for a prolonged period (data not shown), the data suggest that this mode of delivering NAC or other antioxidants might represent an effective adjuvant strategy for reducing posttrauma lung injury.

We tested several different antioxidant formulations. NAC is a hydrophilic antioxidant with multiple functions. The antioxidant activity of NAC is in part due to its ability to donate cysteine groups and thus act as substrate to increase the intracellular levels of the endogenous intracellular antioxidant glutathione.[16] NAC itself can also function as a direct reactive oxygen intermediate scavenger. α-Tocopherol, the major component of vitamin E, has been shown to exert its effects by scavenging reactive oxygen species and by stabilizing membranes containing polyunsaturated fatty acids.[17,18] α-Tocopherol is a sparingly soluble molecule and is too viscous to be delivered directly to the lung. However, it can be incorporated in liposomes, thereby allowing the delivery of considerable amounts of α-T to the lung. Both liposomal NAC and liposomal α-T alone were protective against lung injury, although the effect of liposomal α-T was marginally less than with liposomal NAC. Different sensitivities of ROS to the specific antioxidant used may be one possible explanation for the differential efficacy. Although bifunctional liposomes containing both α-T and NAC may deliver more antioxidant potential, they did not exhibit synergistic or additive effects in the present studies.

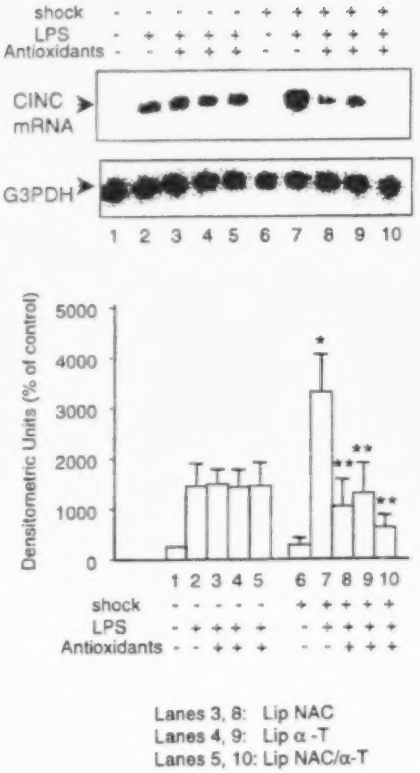


Fig 5. A, Northern blot showing effect of liposomal antioxidants on expression of CINC mRNA extracted from lung tissue of shock and sham animals 22 h after shock/resuscitation and liposomal antioxidants administration (Late protocol) and 4 h after intratracheal LPS. G3PDH mRNA is identified as a control for RNA loading. Blot shown is representative of 4 independent studies. B, Normalized densitometric analysis of CINC mRNA. Data represent the mean ± SEM, n = 4 animals per group. $^{*}P < .05$ vs sham/SAL group; $^{**}P < .01$ vs shock/LPS no antioxidant group.

The data suggest the possibility that soluble NAC and liposomal NAC may be exerting their effects, in part, through different mechanisms. NAC in the resuscitation fluid not only prevented the shock/LPS-induced rise in lung permeability but also reduced lung neutrophil accumulation, consistent with its effect on macrophage chemokine expression (Fig 1). By contrast, although LipNAC

appears to effect a similar reduction in transpulmonary albumin flux, there was only partial reduction in lung PMN sequestration and chemokine expression. These findings suggest that LipNAC may be exerting some of its effects by neutralizing the tissue-toxic oxidant species generated by infiltrating PMNs. In this regard, we have previously shown that the antioxidant pyrrolidine dithiocarbamate completely prevents lung injury without altering PMN influx in an LPS-induced lung injury model.[2] In those studies the lipid soluble dithiocarbamate appeared to protect by preventing lipid peroxidation as assessed by levels of malondialdehyde. Further studies are required to evaluate the cellular mechanisms whereby liposomal antioxidants exert their total effect in this model.

In summary, we demonstrated that liposome encapsulated antioxidants, NAC and α-T, are able to provide sustained protection against ARDS. They appear capable of doing so after hemorrhage/resuscitation by using a low dose of intratracheal direct delivery of liposome encapsulated antioxidants. This system may represent an effective therapeutic approach for the delivery of antioxidants and other anti-inflammatory treatments into the lung after major trauma.

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14. ABSTRACT

(U) Background. We have previously shown that N-acetylcysteine (NAC), an antioxidant, in the resuscitation
fluid after shock prevents lung injury in response to lipopolysaccharide (LPS) by inhibiting chemokine generation by alveolar macrophages in the lung. However, the protection was short-iived. We hypothesized that liposomal (Lip) NAC delivered intratracheally might be delivered directly to the target cells and exert prolonged effect.
Methods. Sprague-Dawley rats were bled to a blood pressure of 40 mm Hg for 1 hour and resuscitated with shed blood and equal volume of Ringer's lactate. In some studies 500 mg/kg NAC was included in the resuscitation fluid. Thirty minutes later, 150 µl LipNAC (9.4 mg/kg NAC) was given intratracheally. One hour and 18 hours after resuscitation, LPS (30 µg/kg) or saline was given intratracheally.
Lung injury was assessed by permeability to 125I-albumin, bronchoalveolar lavage neutrophils and lung myeloperoxidase. The cytokine-induced neutrophil chemoattractant (CINC) expression in the lung was assessed by Northern blot.
Results. At the early time point, both NAC and LipNAC protected the lung with the effects in significantly
reducing the increases in transpulmonary albumin flux, neutrophil influx and myeloperoxidase in the lungs of shock/LPS rats. However, by the late time point, only LipNAC retained its salutary effect. This correlated well with persistent ability to prevent CINC increase. In addition, Lipá-tocopherol (á-T) and LipNAC/á-T were tested and determined to be effective to protect the lung.
Conclusions. Liposomal encapsulation of antioxidants at low dose provides long lasting protection against acute respiratory distress syndrome after shock. This may represent a novel treatment approach.(Surgery 2000;128:332-8.)

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15. KEYWORDS, DESCRIPTORS or IDENTIFIERS

(U) Liposomal Antioxidants; Acute Respiratory Distress Syndrome; Lung Injury.